effectively prevent uptake by the cell, they remove the metal salt only very slowly once it is attached to the cell. Lead phosphate added to cells could, however, be removed by prolonged dialysis against Krebs-Ringer bicarbonate solution containing mm-EDTA (Table 5). Since Foreman & Trujillo (1954) have shown that EDTA cannot enter the erythrocyte, it is clear that the lead removed by the chelating agent must be on the surface of the cell and remain there for at least 24 hr. after the initial uptake. Lead originally present in the cells when the blood specimen was collected could not be removed by this treatment and was presumably located intracellularly. Such intracellular lead, responsible during maturation of the erythrocyte for inhibition of several stages in biosynthesis of haem, is probably in a form other than phosphate, since a peptized phosphate sol would hardly provide sufficient Pb^{2+} ions for inactivation of enzyme.

SUMMARY

1. A study is described of the uptake of lead *in vitro* by human erythrocytes, including experiments on the form of lead in plasma and the kinetics of lead-binding.

2. Lead appears to exist in plasma as a peptized lead phosphate sol, which is rapidly aggregated in a second-order reaction to particulate form on the surface of the erythrocytes. More than 95% of small quantities of added lead are rapidly attached to the cells.

3. No competitive inhibition of uptake of lead was observed with any of a number of potential competitors tested, including other heavy metals, Cu^{2+} , Hg^{2+} , $(UO_2)^{2+}$ and Tl^{4+} ions.

4. Chelating agents such as ethylenediaminetetraacetic acid, hexametaphosphate and glutathione remove lead attached to erythrocytes only slowly *in vitro*; intracellular lead was not withdrawn.

5. It is suggested that the protective effect of plasma and of other solutions containing excess of phosphate is due to a charge effect on the lead phosphate sol, leading to additional negative charge on the erythrocyte.

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Manometric Studies of Bracken [*Pteridium aquilinum* (L.) Kuhn] Thiaminase

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Bracken thiaminase catalyses the fission of the methylene-quaternary nitrogen bond of thiamine with transfer of the pyrimidine moiety to an amine acceptor (see, for example, Fujita, Okamoto & Nose, 1955; Kenten, 1957) according to the general equation

where P and T stand for the pyrimidine and thiazole components of thiamine. Therefore it should be possible to study the transfer reaction of thiaminase manometrically in bicarbonate-carbon dioxide buffer by measuring the carbon dioxide output which would accompany the release of the H^+ ion. In the present work the suitability of the manometric method for studying the action of bracken thiaminase has been examined.

MATERIALS AND METHODS

Plant material and extracts. Bracken fronds were harvested locally in July and August. The pinnae were stripped from the rachis and passed through a domestic meat mincer. The mince was mixed with 1.5 vol. of 0.033M-Na₂HPO₄ and squeezed by hand through strong cotton cloth. Four such extracts were made with thiaminase activities of 66, 42, 20 and 28 units/ml.

Thiaminase preparations. Two preparations were made from fresh bracken. The extracts were filtered through fluted Whatman no. 1 filter papers at 0–4° for 2 days; any extract which had not passed through in this time was clarified by centrifuging at 10 000 *g* for 15 min. and the supernatant added to the filtrate. The method of Kenten (1957) was then followed as far as the second precipitation with (NH₄)₂SO₄. This precipitate was suspended in the minimum amount of 0.01M-phosphate buffer, pH 7.5, and dialysed against several changes of distilled water at 0–4° for 24 hr. The first preparation made from bracken gathered in early July consisted of a dark-brown mobile fluid (enzyme *A*, 0.6 mg. of N/ml.). It contained 49% of the thiaminase present in the extract. The specific activity was 1100, representing a tenfold purification. For the second preparation bracken was gathered in late August. Difficulty was encountered throughout the preparation owing to the presence of large amounts of mucilage. The dialysed second (NH₄)₂SO₄ precipitate (enzyme *B*, 1.28 mg. of N/ml.) had 35% of the activity of the original extract and was highly viscous. Fractionation of part of enzyme *B* with calcium phosphate gel as described by Kenten (1957) gave a light-brown mobile fluid (enzyme *C*, 0.14 mg. of N/ml.) with 55% recovery of its activity. The preparations were stored frozen at –18°, at which temperature no appreciable loss of activity was observed in two months. Apart from the differing specific activities of these preparations, no other differences of activity in the thiaminase-transfer reaction were found.

Enzyme units. One unit of thiaminase activity is that amount of enzyme which catalyses the formation of 1 μmole of *N*-(4-amino-2-methylpyrimidin-5-yl)methylpyridine ion (heteropyrithiamine) in 1 hr. from a mixture of thiamine and pyridine under the standard conditions described by Kenten (1957). The specific activity is the number of enzyme units/mg. of N of the enzyme preparation.

Estimation of thiamine and heteropyrithiamine. After deproteinization of the reaction mixtures with metaphosphoric acid, thiamine was estimated by the method of Melnick & Field (1939) and heteropyrithiamine by the method of Kenten (1957).

Nitrogen and other elements. The nitrogen content of the bracken preparations was determined by a micro-Kjeldahl method and the NH₃ measured by titration with HCl. Other analyses were by Weiler and Strauss, Oxford.

Thiamine chloride hydrochloride. This was of B.P. quality from Roche Products Ltd. Solutions were prepared and adjusted to pH 7.5 with dilute NaHCO₃ immediately before use.

Manometric experiments. These were carried out in the Warburg apparatus. The standard conditions referred to in the text are when the total volume of the reaction mixture was 3 ml., the gas phase CO₂ + N₂ (5:95, v/v), the reactants, with the exception of NaHCO₃, previously adjusted to pH 7.5, the NaHCO₃ concentration 0.05M and the temper-

ature 37°. Under these conditions the pH of the reaction mixtures would be 7.5.

Melting points. These are uncorrected.

Preparation of thiamine analogues

4-Amino-5-bromomethyl-2-methylpyrimidine hydrobromide was prepared by the method of Cline, Williams & Finkelstein (1937); heteropyrithiamine bromide hydrobromide by the method of Kenten (1957); *N*-(4-hydroxy-2-methylpyrimidin-5-yl)methyl-1-5-(β-hydroxyethyl)-4-methylthiazolium chloride hydrochloride (oxythiamine) according to the directions of Rydon (1951); and *N*-(4-amino-2-methylpyrimidin-5-yl)methylaniline by the method of Fujita, Nose, Uyeo & Koizumi (1952b).

N-(4-amino-2-methylpyrimidin-5-yl)methylquinolinium bromide hydrobromide (quinilinothiamine). 4-Amino-5-bromomethyl-2-methylpyrimidine hydrobromide (300 mg.) was dissolved in 12 ml. of isopropyl alcohol and 1 g. of quinoline was added. The mixture was kept at 25° for 6 days. The pale-yellow precipitate was washed with isopropyl alcohol and ether and dried *in vacuo* to give 252 mg., m.p. 234–8° (decomp.). Repeated recrystallization from aqueous methanol gave slightly greenish yellow crystals which, after drying *in vacuo* over conc. H₂SO₄, had m.p. 255–256° (decomp.); Matsukawa & Yurugi (1954) give 261° (decomp.) [Found: Br 37.8. Calc. for C₁₅H₁₆N₄Br₂·½H₂O (Matsukawa & Yurugi found ½ mol. of H₂O): Br 38.0%].

5-(β-Hydroxyethyl)-4-methyl-3-(*o*-nitrobenzyl)thiazolium chloride. 5-(β-Hydroxyethyl)-4-methylthiazole (2 g.) and 2 g. of *o*-nitrobenzyl chloride in 2 ml. of xylene were heated at 100° for 86 hr. The brownish crystalline mass which separated was washed with xylene and ether and dried *in vacuo* to give 3.64 g., m.p. 203–205° (decomp.). Recrystallization from aqueous ethanol and drying *in vacuo* over conc. H₂SO₄ gave pale buff-coloured crystals, m.p. 204–205° (decomp.) (Found: C, 49.2; H, 5.1; Cl, 11.7. C₁₅H₁₅O₃N₂SCl requires C, 49.5; H, 4.8; Cl, 11.3%).

4-Methyl-3-(*o*-nitrobenzyl)thiazolium chloride. This was prepared by a method similar to that of Clarke (1944). 4-Methylthiazole (1.4 g.) and 2 g. of *o*-nitrobenzyl chloride in 2 ml. of xylene were heated at 125° for 24 hr. The crystalline mass of faintly brownish needles was washed with xylene and ether and dried *in vacuo* to give 1.84 g., m.p. 200–201° (decomp.). Recrystallization from ethanol-toluene gave faintly buff-coloured crystals which, after drying *in vacuo* over conc. H₂SO₄, had m.p. 204° (decomp.). Clarke (1944) gives 186.5–187° (decomp.) (Found: N, 10.2; Cl, 12.9. Calc. for C₁₁H₁₁O₂N₂SCl: N, 10.4; Cl, 13.1%).

3-(*o*-Aminobenzyl)-4-methylthiazolium chloride hydrochloride. This was prepared by a method similar to that of Clarke (1944), except that the *o*-nitro compound was reduced by treatment with 3.5 moles of SnCl₂·2H₂O in conc. HCl at 80–90° for 45 min. The procedure of Clarke was then followed. The white crystalline product turned orange at about 190° and then gradually darkened with further increase in temp. up to 270° without melting. Clarke records decomposition over the range 204–212° (Found: Cl, 25.6%; equiv. by titration 281. Calc. for C₁₁H₁₄N₂SCl₂: Cl, 25.6%; mol.wt. 277).

N-(4-Amino-2-methylpyrimidin-5-yl)methylpiperidine. 4-Amino-5-bromomethyl-2-methylpyrimidine hydrobromide (167 mg.) was dissolved in 2.5 ml. of absolute ethanol and 400 mg. of piperidine was added. The mixture was kept at 37° for 24 hr. and then concentrated to dryness over conc.

H_2SO_4 *in vacuo*. The residue was extracted several times with boiling ether and the combined ether extracts were evaporated to dryness. The white crystalline solid (108 mg.) of m.p. 80–84° was recrystallized from aqueous ethanol, to give colourless crystals. Drying *in vacuo* over conc. H_2SO_4 gave an opaque white solid of m.p. 97°, unchanged on further recrystallization and drying *in vacuo* (Found: C, 63.7; H, 8.8; N, 27.0. $\text{C}_{11}\text{H}_{18}\text{N}_4$ requires C, 64.0; H, 8.7; N, 27.2%).

RESULTS

Theoretical basis of the manometric method

In the thiaminase transfer reaction (equation 1) two bases react to give two other bases which may differ considerably from the reactants in the strength of their ionizable groups. The reaction takes place readily at pH 7.5 and if, at this pH, the extent of ionization of the products is less than that of the reactants, acid will be released and in bicarbonate- CO_2 buffer a CO_2 output would be expected to accompany thiaminase action. The following examples make this clear.

(a) If the acceptor amine and the pyrimidinylmethyl-amine product are both very weak bases (pK less than 5.5) the general equation (1) is an exact formulation of the reaction, and at pH 7.5 all the H^+ produced will enter into the equilibrium $\text{H}^+ + \text{HCO}_3^- \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{CO}_2 (\text{dissolved}) \rightleftharpoons \text{CO}_2 (\text{gas})$ (2)

and the evolution of 1 mole of CO_2 would be expected.

(b) If, however, the pyrimidinylmethyl-amine product is a strong base it will be ionized:

and no evolution of CO_2 will be observed. It is possible therefore to have an output of between 0 and 1 mole of CO_2 when the acceptor amine is a weak base.

(c) With a strong base as the acceptor amine the two extreme possibilities may be written:

and an output of between one and two moles of CO_2 would be expected, according to the extent of ionization of the pyrimidinylmethyl-amine product. At pH 7.5 the primary amino group (pK_a 4.5, Williams & Ruehle, 1935) attached to the pyrimidine heterocycle of thiamine will not be ionized, and substitution of a different base for the thiazole moiety would not be expected to lead to an increase in its strength sufficient to cause appreciable ionization at pH 7.5.

Table 1 shows how the expected CO_2 output varies with the pK_a values of the acceptor amine and of the pyrimidinylmethyl-amine product when the thiaminase reaction takes place at pH 7.5.

Total outputs of carbon dioxide with different amines

Preliminary experiments, in which a colorimetric method of estimating thiamine destruction was used, suggested that, of a number of amines which were active in the bracken thiaminase-catalysed transfer reaction, pyridine, aniline, piperidine and trimethylamine were among the most active. These amines, either on account of their difference in ionization at pH 7.5, or because of the degree of ionization of the product they give by the thiaminase reaction, are suitable for testing experimentally a number of the examples discussed above.

The experiments were made under the standard conditions, with 0.3 ml. of enzyme *A* and a concentration of amine of 0.05M. The results are given in Table 2 and Fig. 1. Corrections for CO_2 retention were estimated by adding known amounts of standard acid either to the reaction mixtures at the end of the experiment or to mixtures made up to correspond closely in composition to these final reaction mixtures. The amount of bound CO_2 was found to be small and varied from 7.5 to 11% of the experimental output.

Pyridine. Pyridine is a weak base (pK_a 5.1 at 37°) and the transfer reaction leads to the formation of heteropyrithiamine, which has the quaternary-nitrogen configuration and is a strong base. Therefore the reaction proceeds according to equation (3) and no evolution of CO_2 would be

Table 1. *Relation between the expected output of carbon dioxide and the ionization constants of the acceptor amine and the pyrimidinylmethyl-amine product*

The results are given as moles of CO_2 /mole of thiamine destroyed at pH 7.5.

pK_a of acceptor amine	pK_a of pyrimidinylmethyl-amine product					
	4-5	6	7	8	9	10-11
4-5	1.0	0.97	0.75	0.25	0.03	0
6	1.03	1.0	0.78	0.28	0.06	0.03
7	1.25	1.22	1.0	0.50	0.28	0.25
8	1.75	1.72	1.50	1.0	0.78	0.75
9	1.97	1.94	1.72	1.22	1.0	0.97
10-11	2.0	1.97	1.75	1.25	1.03	1.0

expected. Experimentally no CO_2 output was found in a period of 1 hr., although estimation of heteropyrithiamine in the reaction mixtures showed that 14.1 μmoles had been formed from the

Fig. 1. Dependence of the CO_2 output on the amounts of thiamine and piperidine present. The experiments were made under the standard conditions. When thiamine was varied, the reaction mixtures contained 0.05 M-piperidine and 0.3 ml. of enzyme A and 2.95 ($\times$), 5.95 ($\circ$), or 8.9 ($+$) μmoles of thiamine. When piperidine was varied, 0.5 ml. enzyme was used, the thiamine concn. was 0.005 M and the mixtures contained 4 ($\square$) or 7 ($\triangle$) μmoles of piperidine. $\blacksquare$, Enzyme heated for 15 min. at 100° before use, 8.9 μmoles of thiamine and 0.05 M-piperidine, $\bullet$, 8.9 μmoles of thiamine, piperidine omitted. The values are corrected for the blank outputs when thiamine was omitted, but not for CO_2 retention. The arrows show the outputs in terms of 1 mole of CO_2 /mole of the limiting reactant.

14.8 μmoles of thiamine added. No thiamine was detected in these mixtures at the end of the reaction.

Aniline. Titration of synthetic *N*-(4-amino-2-methylpyrimidin-5-yl)methylaniline (the expected product) with dilute hydrochloric acid at the glass electrode gave pH 6.6 as the mid-point of the neutralization curve of the aniline nitrogen group at 22° . By applying the temperature correction of Hall & Sprinkle (1932) the pK_a of the aniline nitrogen group at 37° would be about 6.3 and at pH 7.5 it would be partly ionized. Since aniline is a weak base (pK_a 4.4 at 37°) an output of 0.94 mole of CO_2 would be expected from the thiaminase reaction, according to equations (1) and (3). Experimentally a mean value of 0.89 mole of CO_2 /mole of thiamine destroyed was obtained.

Piperidine. Piperidine is a moderately strong base (pK_a 10.8 at 37°) and it would be fully ionized at pH 7.5. The pK_a of the piperidine nitrogen group of the pyrimidinylmethylpiperidine product was estimated in the same way as for the aniline compound and found to be about 8 at 37° . The thiaminase reaction would therefore be expected to lead to the evolution of 1.25 moles of CO_2 /mole of thiamine destroyed, according to equations (4) and (5) and Table 1. A mean value of 1.15 moles of CO_2 was obtained experimentally. It can be seen from the general equation (1) that either the amount of thiamine or the amount of amine present can limit the reaction. It was found that when thiamine was present in excess the CO_2 outputs obtained were slightly greater, the mean value being 1.25 moles of CO_2 /mole of piperidine added.

Trimethylamine. Trimethylamine (pK 9.5 at 37°) would be 99% ionized at pH 7.5; since the product of the thiaminase reaction would have the quaternary-nitrogen configuration, a CO_2 evolution of 0.99 mole/mole of thiamine destroyed would be expected, from equations (3) and (4). With thiamine

Table 2. Total outputs of carbon dioxide with different amines

The experimental conditions were similar to those of Fig. 1 except that either enzyme A or C was used. When the amine was present in excess, 2.95, 5.95 or 8.9 μmoles of thiamine were added. When thiamine was present in excess its concentration was 0.005 M and 4 or 7 μmoles of piperidine were added. The values are corrected for CO_2 retention. Blank uptakes obtained when thiamine was omitted have been subtracted. The evolution of CO_2 had stopped in all experiments after 4–5 hr.

Amine present	Amine concn. (M)	Total output of CO_2 (moles/mole of thiamine destroyed)	
		Expected	Found
Pyridine	0.05	0	0
Aniline	0.05	0.94	0.89
Trimethylamine	0.05 or 0.1	0.99	0.90
Piperidine	0.05	1.25	1.15
(Piperidine; thiamine in excess)	—	(1.25)*	(1.25)*

* Moles of CO_2 /mole of piperidine added.

as limiting substrate the mean value found experimentally was 0.90 mole of CO_2 . Experiments with trimethylamine in limiting amount were not attempted because of the rapid decrease in reaction velocity with decreasing trimethylamine concentration (Table 4).

The results show that with piperidine, aniline and trimethylamine rather less than the expected CO_2 output was obtained. The pK_a values for the products used for calculating the CO_2 outputs when piperidine and aniline are the acceptors are only approximate and may account for part of the discrepancy. With trimethylamine there is no such uncertainty; yet, when thiamine was limiting, low values for the total CO_2 output were also obtained. Such results could be accounted for if the samples of thiamine used were impure. However, recrystallization of the thiamine from methanol-ethanol failed to alter the amount of CO_2 evolved. Further, when pyridine was used as acceptor, about 0.95 mole of the expected product, heteropyrithiamine, was formed per mole of thiamine destroyed, suggesting that the thiamine was at least 95% pure. A possible reason for the low outputs is that a small amount of thiamine is destroyed under the manometric conditions by a mechanism that does not lead to the release of H^+ ion. This is supported by the results obtained when thiamine was present in excess and piperidine was limiting, for somewhat higher results were then obtained.

Proof that the transfer reaction is the major one under the manometric conditions was given by the isolation, in reasonably good yield, of the pyrimidinylmethylpiperidine product from a large-scale reaction mixture.

To 150 mg. of thiamine chloride hydrochloride, 13.5 ml. of 0.2M- NaHCO_3 and 4.5 ml. of m-piperidine hydrochloride (pH 7.5) in a total volume of 86 ml., enzyme A (4 ml.) was added. The mixture was gassed with $\text{N}_2 + \text{CO}_2$ (95:5, v/v), a few drops of CHCl_3 were added towards the end of the gassing, and the vessel was sealed. After the mixture had been kept at 37° for 8 hr., 10 ml. of 50% (w/v) metaphosphoric acid was added and the precipitate removed by centrifuging. The supernatant was made strongly alkaline with 10N- NaOH and extracted continuously with ether for 24 hr. The ether extract was evaporated to dryness *in vacuo*. The pale-yellow residue was recrystallized from aqueous ethanol, to give colourless crystals which dried *in vacuo* over conc. H_2SO_4 , forming a white solid. The m.p. was 96°. The mixed m.p. with an authentic sample of N-(4-amino-2-methylpyrimidin-5-yl)methylpiperidine (m.p. 97°) was 97°. The weight was 43.3 mg., representing a 53.5% yield, based on the thiamine added.

Effect of varying different factors on the activity of bracken thiaminase

Different amines. The activity of a number of different amines in the transfer reaction catalysed by bracken thiaminase has been tested by the

manometric method and the results are shown in Table 3. A number of the amines tested are known plant constituents, and with the exception of ammonia and m-aminobenzoic acid, which gave inconclusive results, they were all active in the transfer reaction. The extent of CO_2 binding which might be brought about by the presence of amines and hence lead to a low output was not determined

Table 3. *Effect of different amines on the rate of the thiaminase reaction*

The standard conditions were used (see Materials and Methods). The thiamine concentration was 0.005M and the amines, previously adjusted to pH 7.5 with HCl, were present at a final concentration of 0.05M. Thiaminase preparation A (0.3 ml.) was used as enzyme. The values given are corrected for the blank outputs obtained when thiamine was omitted or when enzyme previously heated at 100° for 15 min. was used. The blank values did not exceed 8 $\mu\text{l.}$ in 1 hr. The values are not corrected for CO_2 retention.

Amine	Output of CO_2 in 1 hr. ($\mu\text{l.}$)
Trimethylamine	280
Triethylamine	12.5
Piperidine	160
N-Methylpiperidine	13
Diethylamine	100
Aniline	152
Tyramine	130
Ethanolamine	121
Taurine	98
Tryptamine	78
m-Aminobenzoic acid	3
Putrescine	26
Ammonia	0

Table 4. *Effect of varying the concentration of piperidine and trimethylamine on the rate of the thiaminase reaction*

The standard conditions were used (see Materials and Methods). The thiamine concentration was 0.005M and the amine concentration was varied. Thiaminase preparation A was used as enzyme, 0.3 ml. with piperidine and 0.1 ml. with trimethylamine. The reaction velocity was calculated from the output in the first 30 min. of the reaction. Blank uptakes when thiamine was omitted or when heated enzyme (15 min. at 100°) was used were less than 3 $\mu\text{l.}$ of CO_2 in 30 min. Values are corrected for CO_2 retention.

Concn. of amine (M)	Output of CO_2 ($\mu\text{l./hr.}$)	
	Piperidine	Trimethylamine
0	0	0
0.0025	68.5	3.5
0.01	127	48
0.025	156	—
0.05	163	187.5
0.075	156	—
0.2	132	—
0.3	—	570
0.5	103	—

experimentally with all of the amines. With aniline, piperidine and trimethylamine the values for retention of CO_2 did not exceed 11% and it is unlikely that values much greater than this would have been obtained for the other amines.

Amine concentration. The effect of varying the concentration of piperidine and trimethylamine on

Table 5. *Effect of varying the thiamine concentration on the rate of the thiaminase reaction*

The standard conditions were used. The piperidine concentration was 0.05M and the thiamine concentration was varied. Thiaminase preparation A (0.2 ml.) was used as enzyme. The reaction velocity was calculated from the output in the first 30 min. of the reaction. Blank uptakes when thiamine or piperidine were omitted were less than 2.5 $\mu\text{l.}$ in 30 min. The values are not corrected for CO_2 retention but from the results of the other experiments this would not be greater than about 11%.

Concn. of thiamine (M)	Output of CO_2 ($\mu\text{l./hr.}$)
0.001	68.5
0.002	86
0.004	105.5
0.006	107
0.008	116
0.016	123
0.024	129

Fig. 2. Effect of varying the amount of enzyme on the rate of evolution of CO_2 . The standard conditions were used with 0.005M-thiamine. x, Piperidine (0.05M) and enzyme A; +, trimethylamine (0.05M) and enzyme A diluted with 1 vol. of water. The rate of CO_2 evolution was constant over the first 20-25 min. of the reaction and it is from measurements in this period that the rates plotted were calculated. The values are corrected for the blank uptakes (1-6 $\mu\text{l./hr.}$) when thiamine was omitted. The values are not corrected for CO_2 retention, but experience with similar mixtures suggests that such retention would not be greater than 10%.

the velocity of the reaction is shown in Table 4. With piperidine, the results show that the velocity increases with increase in piperidine concentration up to about 0.025M but decreases with relatively high piperidine concentrations. The results also show that the velocity of the reaction falls off with decrease in trimethylamine concentration much more sharply than with decrease in piperidine concentration.

Thiamine concentration. The effect of varying the thiamine concentration with piperidine as acceptor is shown in Table 5. The results show that comparatively large increases in reaction velocity follow an increase in thiamine concentration up to about 0.005M but that further increase in thiamine concentration brings about only a small increase in the velocity.

Effect of varying the amount of enzyme. Under the standard conditions, with either piperidine or trimethylamine as acceptor, the rate of evolution of CO_2 is directly proportional to the amount of enzyme used, provided that the velocity does not exceed about 350 $\mu\text{l.}$ of $\text{CO}_2/\text{hr.}$ (Fig. 2). The rates of evolution of CO_2 with piperidine and trimethylamine are 24 and 78 $\mu\text{moles/ml.}$ of enzyme A/hr. respectively. With pyridine as acceptor, under the conditions used by Kenten (1957) for the estimation of thiaminase activity, the rate of formation of heteropyrithiamine was 650 $\mu\text{moles/ml.}$ of enzyme A/hr. The activity of enzyme A with pyridine as acceptor is therefore some 8 times that with trimethylamine and about 27 times that with piperidine. By using some of the thiaminase preparations of Kenten (1957) it was found that, on average, the rate with pyridine was 26 times that with piperidine manometrically.

Activity of bracken thiaminase towards thiamine analogues. The activity of bracken thiaminase towards a number of compounds having structural similarities to thiamine has been tested manometrically and the results are given in Table 6. Piperidine was used throughout as the acceptor amine. In calculating the expected output when compounds other than thiamine are tested as substrates, the same considerations apply as those discussed previously, namely that it is the extent of ionization of the acceptor amine and the products, as well as their alteration in concentration, which will control the CO_2 output. Heteropyrithiamine and quinilinothiamine differ from thiamine in that the thiazole residue is replaced by pyridine and quinoline respectively. Hence with piperidine (pK_a 10.8 at 37°) as acceptor the transfer reaction would give the same pyrimidine-methylpiperidine compound as that obtained with thiamine, but, instead of a thiazole compound, pyridine or quinoline would be released. Since neither pyridine nor quinoline would be significantly ionized at pH 7.5,

the thiaminase reaction should lead to an output of 1.25 moles of CO_2 /mole of heteropyrithiamine or quinilinothiamine destroyed (see Table 1). With oxythiamine, which differs from thiamine in that the primary amino group attached to the pyrimidine residue is replaced by hydroxyl, similar considerations apply. With the benzyl compounds, the pK_a of the benzylpiperidine compounds which would be formed if thiaminase action took place is not known; if they were very weak bases an output of 2 moles of CO_2 would be expected [equation (5)], and if strong bases, 1 mole of CO_2 /mole of benzylthiazolium compound destroyed [equation (4)]. The failure to demonstrate thiaminase activity towards the benzylthiazolium compounds is therefore almost certainly because they are not attacked by the enzyme under the conditions used. It is also clear that both quinilinothiamine and pyrithiamine are readily attacked by the enzyme in the presence but not in the absence of piperidine, suggesting that, as with thiamine (Kenten, 1957), the enzyme does not easily catalyse the hydrolytic fission of these compounds. With oxythiamine a small but definite activity was found. The activity was the same with two different preparations of oxythiamine and was unchanged after these had been recrystallized twice. Rydon (1951) reports that oxythiamine, made by his method, contains not more than 0.00015% of thiamine, which amount would be insufficient to give a measurable

output of CO_2 under the conditions used with oxythiamine.

At a concentration of 0.05M-piperidine the maximum rate of thiaminase action is nearly reached at 0.005M-thiamine (Table 5). Under these conditions the addition of either 0.005M-heteropyrithiamine or 0.005M-quinilinothiamine failed to bring about an appreciable increase in the rate of evolution of CO_2 , suggesting that thiaminase is involved in the attack on all three compounds. Similarly the presence of oxythiamine in addition to thiamine did not affect the rate of evolution of CO_2 ; this suggests that oxythiamine has a low affinity for the enzyme, but because of the low activity with oxythiamine alone this cannot be used as evidence that thiaminase attacks oxythiamine. The presence of the *o*-nitrobenzylthiazolium compounds was without appreciable effect on the activity of thiaminase towards thiamine, but the *o*-aminobenzylthiazolium compound (ABMT) inhibited strongly. Under the conditions used in Table 6 the inhibition by this compound increased with time until, after about 30 min., it was virtually complete.

The effect of ABMT on the activity of bracken thiaminase is further illustrated in Fig. 3. The results show that if the enzyme preparation is incubated with the inhibitor before the thiamine is added the amount of inhibition obtained in the first 20–30 min. of the reaction is increased. How-

Table 6. *Activity of bracken thiaminase towards thiamine analogues*

The standard conditions and enzyme B (0.55 ml.) or C (0.4 ml.) were used. The piperidine concn. was 0.05M and thiamine and thiamine analogue concn. 0.005M. When the effect of the thiamine analogues on the rate of reaction with thiamine was tested, with the exception of compound E, which was used at 0.0005M, both compounds were present at 0.005M final concn. The values are corrected for the blank uptakes when thiamine or analogue was omitted (4–7 $\mu\text{l.}$). Heating the enzyme for 15 min. at 100° abolished over 95% of the activity towards compounds A, B, C and D. The values are not corrected for CO_2 retention; this was determined with some of the systems and did not exceed 10% of the experimental output.

Compound	Output of CO_2		
	With piperidine		Without piperidine
	($\mu\text{l.}$ of CO_2 /hr.)*	($\mu\text{l.}$ of CO_2 in 3 hr.)	($\mu\text{l.}$ of CO_2 in 3 hr.)
A Thiamine	149	293	5
B Heteropyrithiamine	86	191	3
C Quinilinothiamine	80	198	0
D Oxythiamine	10	27	3.5
E 3-(<i>o</i> -Aminobenzyl)-4-methylthiazolium chloride	—	3	—
F 4-Methyl-3-(<i>o</i> -nitrobenzyl)thiazolium chloride	—	1.5	—
G 5-(β -Hydroxyethyl)-4-methyl-3-(<i>o</i> -nitrobenzyl)thiazolium chloride	—	2.5	—
A + B	158	—	—
A + C	149	—	—
A + D	141	—	—
A + E (0.0005M)	30†	—	—
A + F	159	—	—
A + G	138.5	—	—

* Calculated from measurements in the first 30 min. of the reaction.

† Evolution of CO_2 in first hr.

ever, this difference diminishes with time and after 20–30 min. the rates of reaction are about the same whether the enzyme has, or has not, been previously incubated with the inhibitor. ABMT is a powerful inhibitor of the bracken-thiaminase reaction. Under the conditions of Fig. 3, when the enzyme preparation was incubated with the inhibitor at $2\mu\text{M}$ before thiamine was added the reaction velocity was diminished by 15–20%; at a concentration of $50\mu\text{M}$ the inhibition was almost complete. Increasing the concentration of thiamine was without appreciable effect on the amount of inhibition (Table 7).

DISCUSSION

The present work shows that the manometric method is suitable for studying the action of bracken thiaminase. The calculated data of Table 1 show that for most systems an evolution of

Fig. 3. Effect of 3-(*o*-aminobenzyl)-4-methylthiazolium chloride (ABMT) on the activity of bracken thiaminase. The experiments were made under the standard conditions with a thiamine concn. of 0.005M . In one experiment the enzyme *A* (0.05 ml.) was incubated with ABMT in the presence of 0.1M -trimethylamine and 0.05M - NaHCO_3 in the main vessel for 20 min. at approx. 20° and then for 15 min. at 37° before the thiamine (0.005M final concn.) was added from the side arm. In the other experiment thiamine (0.005M), trimethylamine (0.1M), NaHCO_3 (0.05M) and ABMT were in the main vessel and enzyme *A* (0.05 ml.) was added from the side arm after 15 min. at 37° . Enzyme added from the side arm; ●, no ABMT; ■, $10\mu\text{M}$ -ABMT; ▲, $50\mu\text{M}$ -ABMT. Enzyme incubated with ABMT, thiamine added from side arm; ○, no ABMT; +, $2\mu\text{M}$ -ABMT; □, $10\mu\text{M}$ -ABMT; △, $50\mu\text{M}$ -ABMT. The values given are corrected for blank uptakes in the absence of thiamine; these were less than $7.5\mu\text{l.}$ in 90 min. The values are corrected for CO_2 retention, which was found to be 7% with the system containing the largest amount of ABMT.

CO_2 would be expected as a result of thiaminase action. The quantitative studies with aniline, pyridine, piperidine and trimethylamine suggest that, although (under the conditions used when thiamine was limiting) some 5–10% of the thiamine may be lost in side reactions, there is a reasonable agreement between the calculated and experimental outputs of CO_2 . Sealock & Livermore (1944) demonstrated that an output of CO_2 followed the action of a preparation of thiaminase from carp viscera on thiamine in bicarbonate solution. They obtained outputs varying from 0.57 to 0.86 mole of CO_2 /mole of thiamine destroyed. With the exception of the thiaminase produced by a bacterium (Fujita, Nose & Kuratani, 1954) which catalyses the hydrolytic fission of the methylene-quaternary nitrogen bond of thiamine, most of the thiaminases have been shown to catalyse the transfer reaction of equation (1). It appears probable therefore that the manometric method may prove useful in studies of other thiaminases.

In the present work it has been shown that a number of amines, many of which are known plant constituents, are active in the bracken-thiaminase-catalysed transfer reaction. Although Fujita *et al.* (1952*a*) found that taurine failed to increase the rate of thiamine destruction by crude preparations of thiaminase from shellfish, fish and bacteria, the present work shows (Table 3) that this amino compound is reasonably active with bracken thiaminase. When thiamine is added to an aqueous suspension of ground Quahog clams, thiaminase action leads to the formation of ictthiamine

Table 7. Effect of varying the thiamine concentration on the inhibition of bracken thiaminase by 3-(*o*-aminobenzyl)-4-methylthiazolium chloride (ABMT)

The conditions used were those of Fig. 3, the enzyme being incubated with $4\mu\text{M}$ -ABMT in the main vessel along with the other reactants for 30 min. at room temp. and then for 15 min. at 37° before adding thiamine from the side arm. Under these conditions the rate of evolution of CO_2 was almost constant over the first 20 min. of the reaction and the values given below were calculated from measurements in this period. The values are not corrected for CO_2 retention. With boiled enzyme and mixtures corresponding to the highest thiamine concentration, CO_2 retention was estimated to be 7%. The values given are corrected for the rates in the absence of thiamine, these were less than $6\mu\text{l./hr.}$

Concn. of thiamine (mm)	Output of CO_2 ($\mu\text{l./hr.}$)		Inhibition (%)
	Without inhibitor	With inhibitor	
0.99	132	72	45.5
1.48	166.5	78	53
2.46	183	94	49.5
6.17	214.5	98	54

(Barnhurst & Hennessy, 1952). It is likely that ictiamine is a pyrimidine-taurine compound produced by a thiaminase-transfer reaction involving taurine as the natural acceptor amine (Woolley, 1953).

Kenten (1957) proposed, by analogy with the mechanism which is thought to hold for the other transferring enzymes, that the bracken thiaminase reaction could be formulated:

If this mechanism is correct it might be expected that amines which were not ionized would react more readily with the positively charged pyrimidine-enzyme complex. It is surprising therefore that, for example, piperidine ($\text{p}K_a$ 10.8), which would be fully ionized under the experimental conditions, was as active as aniline ($\text{p}K_a$ 4.4).

In agreement with the results of Kenten (1957) no evidence was obtained that the bracken-thiaminase preparations used were capable of catalysing the hydrolytic fission of thiamine. Under the conditions used such activity would have led to the evolution of CO_2 :

In the absence of an acceptor amine the CO_2 outputs obtained were extremely small and it must be concluded that if hydrolytic activity were present it must be negligible in comparison with the transfer activity of the preparations.

It is shown that with both trimethylamine and piperidine as acceptor amines, the rate of evolution of CO_2 is directly proportional to the amount of bracken thiaminase preparation added. Although with these amines under the manometric conditions the thiaminase activity is less than when pyridine is used as acceptor under the conditions of Kenten (1957), it appears that the manometric method should be of use for the estimation of thiaminase activity.

Inhibition of thiaminase by ABMT would be expected to be competitive because of its structural resemblance to thiamine. With fish thiaminase Sealock & Goodland (1944) have shown that ABMT is a competitive inhibitor. The extent of inhibition, however, was found to vary with the particular thiaminase preparation used and a second factor, possibly Mn^{2+} ion, was implicated in the binding of the inhibitor to the enzyme (Sealock & Livermore, 1949). With bracken thiaminase, ABMT inhibits very strongly but the inhibition is not competitive, for, increasing the concentration of thiamine fails to decrease the amount of inhibition. Also, the amount of inhibition increases with increase in time of exposure of the thiaminase to ABMT. It is possible that the ABMT initially

combines reversibly with the enzyme at the same site as thiamine but that a second irreversible reaction follows which gives an inactive ABMT-enzyme complex. Another possibility is that under the experimental conditions ABMT is altered in some way to give a powerful, but non-competitive, inhibitor of bracken thiaminase.

Fujita *et al.* (1955) have shown that heteropyrithiamine and quinilinothiamine are substrates of bracken thiaminase. They demonstrated the formation of thiamine when these compounds were incubated with the thiazole component of thiamine as acceptor amine in the presence of bracken thiaminase. The reaction did not proceed readily under the conditions used by Fujita *et al.*, and after 40 hr. at 50° only 1–3 % of the expected amount of thiamine was formed. In the present work it is shown that both heteropyrithiamine and quinilinothiamine are readily attacked by bracken thiaminase and that this enzyme may also have a slight activity towards oxythiamine.

These results show that the manometric method can be used to test the activity of thiaminase towards structural analogues of thiamine. The method is therefore likely to be of value in testing the action of thiamine on compounds for which analytical methods are not available.

An advantage of the manometric method is that the variation of the rate of the reaction with time can be kept under continuous observation. With other methods this would involve the analysis of a great many samples. The method does suffer from the disadvantage that only a small range of pH can be usefully studied with bicarbonate-carbon dioxide buffer, and a further disadvantage is that the ionization constants of the reactants and products must be known in order to calculate the expected evolution of CO_2 .

The bearing of the present results on thiaminase action *in vivo* is not clear since its function is not understood. The possible physiological functions of thiaminase have been discussed by Woolley (1953) and Kenten (1957).

SUMMARY

1. The transfer reaction of bracken thiaminase can lead to the production of H^+ ions. Under these conditions it is shown that the reaction can be studied manometrically in bicarbonate-carbon dioxide buffer.

2. When aniline, piperidine, pyridine and trimethylamine are used as acceptor amines with thiamine in limiting amount, about 5–10 % of the thiamine may be lost in side reactions, but there is a reasonable agreement between the calculated and experimental carbon dioxide output.

3. The effect on the rate of bracken thiaminase action of varying the concentration of thiamine

and the concentration and type of acceptor amine has been studied.

4. The manometric method has been used to demonstrate that heteropyrithiamine, quinilinothiamine and possibly oxythiamine are thiaminase substrates.

5. The effect of several structural analogues of thiamine on bracken thiaminase has been studied and it is shown that 3-(*o*-aminobenzyl)-4-methylthiazolium chloride is a powerful but non-competitive inhibitor.

6. Conditions are described under which the rate of evolution of carbon dioxide is directly proportional to the amount of bracken-thiaminase preparation present. It is suggested that the manometric method could be used for estimating thiaminase activity.

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Uridine Diphosphoglucose Pyrophosphorylase of Pea Seeds

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The reaction (1) catalysed by uridine diphosphoglucose (UDPG) pyrophosphorylase involves the pyrophosphorolytic cleavage of UDPG with the formation of uridine triphosphate (UTP) and glucose 1-phosphate (G 1-P):

The pyrophosphorolysis of UDPG was first reported by Munch-Petersen, Kalckar, Cutolo & Smith (1953) in *Zwischenferment* preparations. UDPG pyrophosphorylase has also been found in extracts from liver (Mills, Ondarza & Smith, 1954) and mammary gland (Smith & Mills, 1955). A more detailed investigation of the properties of the enzyme was carried out by Munch-Petersen (1955) with a preparation from yeast. Burma & Mortimer (1956) obtained evidence that UDPG pyrophosphorylase was present in homogenates of sugar-beet leaves and Neufeld, Ginsburg, Putman, Fanshier & Hassid (1957) have shown that the enzyme is present in extracts from mung bean and several other plant tissues.

Experiments on the synthesis of sucrose from G 1-P and fructose had suggested that UDPG pyrophosphorylase was active in pea extracts (Turner, 1953, 1954, 1957). In the present investigation UDPG pyrophosphorylase was found in pea-seed extracts and a preparation free from interfering enzymes was obtained. Some properties of the enzyme were studied and its presence in certain other plant tissue extracts was established. The equilibrium of the reaction was examined.

MATERIALS AND METHODS

Substrates. UTP was obtained from Pabst Laboratories, Milwaukee, Wis., U.S.A.

UDPG was obtained from Pabst Laboratories and from Sigma Chemical Co., St Louis, Mo., U.S.A.

G 1-P was prepared by an unpublished method of Professor C. S. Hanes and Dr R. Hill.

Sodium pyrophosphate was analytical-reagent grade (British Drug Houses Ltd.).

Preparation of uridine diphosphoglucose pyrophosphorylase from pea seeds. A crude extract was prepared from dried pea seeds (*Pisum sativum* L.) as described by Turner (1957), and centrifuged at 20 000 g for 20 min. at room